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The Fragile X Phenotype in a Mosaic Male With a Deletion Showing Expression of the *FMR1* Protein in 28% of the Cells

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The instability of the CGG repeat region of *FMR1* is not restricted to the CGG repeat but expands to flanking sequences as well. A mosaic fragile X male is reported with a deletion of part of the CGG repeat and 30 bp immediately 3′ of the repeat, thus confirming the presence of a hotspot for deletions in the CGG region of FMR1. The deletion, detected in 28% of his lymphocytes, did not impair the transcription and translation of *FMR1*, suggesting that regulatory elements are not present in the deleted region. The patient has the characteristic fragile X phenotype and assuming that the mosaic pattern detected in the lymphocytes reflects the mosaic pattern in brain, 28% expression of *FMRP* may not be sufficient for normal cognitive functioning. © 1996 Wiley-Liss, Inc.

KEY WORDS: fragile X syndrome, mosaic male patient, FMRP expression

INTRODUCTION

The fragile X syndrome is one of the most common forms of inherited mental retardation. In most patients, the disease is associated with a large expansion of a CGG trinucleotide repeat (>200 CGGs) in the 5′ untranslated region of the *FMR1* gene [Verkerk et al., 1991; Oberlé et al., 1991; Yu et al., 1991]. This full mutation coincides with hypermethylation of both the CGG repeat and the *FMR1* CpG island [Hansen et al., 1992; Sutcliffe et al., 1992; Hornstra et al., 1993], resulting in the repression of *FMR1* mRNA [Pieretti et al., 1991] and *FMR1* protein (*FMRP*) expression [Verheij et al., 1993; Devys et al., 1993]. A smaller expansion of 50–200 CGGs, called a premutation, can be detected in unaffected male and female carriers and is not associated with abnormal methylation [Fu et al., 1991a; Reiss et al., 1994].

Instability of the expanded CGG repeats occurs both meiotically, leading to differences in repeat lengths between parents and offspring, and mitotically, resulting in a variety of repeat lengths within an individual. The full mutation in patients is visible as a smear on Southern blot analysis, thus displaying a variety of repeat lengths. Some patients are mosaic for a full mutation in combination with either a premutation [Oberlé et al., 1991; Rousseau et al., 1991; Nolin et al., 1994] or normal allele [Van den Ouweland et al., 1994] or deletion [De Vries et al., 1993; De Graaff et al., 1995a] in a proportion of their cells. Moreover, it has been demonstrated that in male patients with a full mutation in their blood cells, a premutation is present in their sperm cells [Reyniers et al., 1993].

The absence of *FMRP* expression is generally accepted to be the cause of the fragile X syndrome. Indeed, all males with a methylated full mutation are mentally retarded, whereas in approximately 60% of the female carriers mental impairment is observed [Rousseau, et al., 1991, 1994a]. Since the premutation, which is not methylated, results in a normal *FMRP* expression, a less severe phenotype in mosaic patients is expected. However, males mosaic for either a full mutation and a premutation do not have a significantly different clinical phenotype than patients with a methylated full mutation only [Rousseau et al., 1994a; De Vries et al., 1993]. Recent reports have described methylation mosaics having an IQ ranging from normal to mental retardation [McConkie-Rosell et al., 1993; Hagerman et al., 1994; Rousseau et al., 1994b; Smeets et al., 1995].

In this paper we describe a male fragile X patient mosaic for a methylated full mutation and an allele with a deletion of part of the CGG repeat and 30 bp immediately 3′ of the CGG repeat. The deletion did not impair transcription and translation of FMRP, indicating the absence of regulatory elements in the deleted region. Despite the presence of *FMRP* in 28% of the blood lymphocytes, this male displayed the clinical phenotype of the fragile X syndrome.

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MATERIALS AND PATIENTS

Clinical Report

This 68-year-old man (J) was moderately retarded, normally proportioned, and had a height of 168 cm (10th centile), a head circumference of 54 cm (25th centile), long face with large anteverted ears, blue pale irises, flat feet, broad halluces, a soft velvety skin, and macroorchidism (50 ml/50 ml). Further, he showed the typical behavior, such as gaze avoidance, perseverative speech, and echolalia, as is frequently observed in fragile X patients.

Southern Blot

Genomic DNA was extracted from blood leukocytes according to standard procedures [Miller et al., 1988]. Seven micrograms DNA was digested to completion with *Hin*dIII alone or in combination with *Eag*I, electrophoresed on a 0.7% agarose gel, and transferred to Hybond N+ blotting membrane. Probe pP2, a 1 kb *Pst*I fragment derived from pE5.1 which detects the (CGG)n repeat and the preceding CpG island [Oostra et al., 1993], was used after labelling by the random oligonucleotide priming method [Feinberg and Vogelstein, 1983]. After overnight hybridization the filters were washed to 1×SSC, 0.1% SDS at 65°C prior to exposure to X-ray film.

PCR Analysis

Amplification was performed using 200 ng of DNA in the presence of 10% DMSO, 0.2 mM each of dATP, dCTP, and dTTP, 0.05 mM dGTP, 0.15 mM 7-deaza-dGTP, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 1 mM MgCl, 1.25 U *Taq* polymerase (BRL), and 1 μM of each primer *a* and *258*. The reactions were first denatured for 5 min at 95°C, followed by 32 cycles of 45″ 95°C and 2½ min 68°C. A final extension of 5 min was carried out at 68°C. After a Centricon-100 purification step (Amicon), the PCR products were directly sequenced with primer *c* and *f* using the Exo(-) Pfu Cyclist DNA Sequencing Kit (Stratagene). The following primers were used (positions in the pE5.1 sequence are indicated between brackets [Fu 1991b]:

a: 5′ GGAACAGCGTTGATCACGTGACGTGGTTTT (2524-2553)

c: 5′ GCTCAGCTCCGTTTCGGTTTCACTTCCGGT (2599-2628)

f: 5′ AGCCCCGCACTTCCACCACCAGCTCCTCCA (2838-2867)

258: 5′ GGGGCCTGCCCTAGAGCCAAGTACCTTGT (2883-2911)

Immunocytochemistry

Blood smears were made from one drop of blood within 6 hr after bleeding. Slides were air-dried and fixed with 3% paraformaldehyde for 10 min followed by 20 min permeabilization in 100% methanol. Immunolabeling was performed with monoclonal antibodies 1a1 against *FMRP* (kindly provided by J.L. Mandel) as described recently for blood smears [Willemsen et al., 1995]. In every preparation 100 lymphocytes were examined and scored for the presence of *FMRP*.

If blood smears could not be made within 6 hr after bleeding, blood samples were stored overnight at 4°C. Lymphocytes were cultured for 3 days according to standard procedures [Verkerk et al., 1992] and washed with PBS. Cytospins were made on slides, which were subsequently fixed and immunolabeled as described above.

RESULTS

Patient J was encountered during an ongoing screening program in which the incidence of the fragile X syndrome in institutes for the mentally disabled is evaluated. Screening for the fragile X mutation was initially performed by PCR with primers c and f. If no normal band was detected, the screening was continued using standard Southern blot analysis with probe pP2 and *Hin*dIII and *Hin*dIII-*Eag*I digests. In normal individuals, digestion of the DNA with *Hin*dIII alone results in a 5.2 kb fragment (Fig. 1, lane 2). In patient J a mosaic pattern was found: beside a full mutation smear of over 6.5 kb an additional fragment was detected representing 20–30% of the DNA (Fig. 1 lane 1). This fragment appeared to be slightly smaller than 5.2 kb. The reduction in size was confirmed by using *Hin*dIII-*Eag*I double digests. Whereas the normal unmethylated *Hin*dIII-*Eag*I band is 2.8 kb (lane 4), the fragment in the patient was approximately 2.7 kb, indicating the presence of a 100 bp deletion within this fragment. The finding that the fragment was digested by *Eag*I implies that the fragment was not methylated, this in contrast to the full

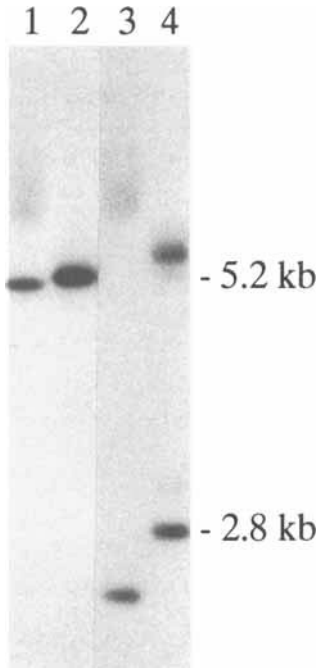

Fig. 1. Southern blot analysis of the patient. DNA was digested with *Hin*dIII alone (**lanes 1,2**) or in combination with *Eag*I (**lanes 3,4**). Following electrophoresis, fragments were blotted on filters and hybridized with pP2. Lanes 1 and 3 represent patient J, lane 2: female control; lane 4: female carrier of a premutation. The normal lengths of the fragments are indicated.

mutation which remained a smear of over 6.5 kb (Fig. 1, lane 3).

To study the location of the deletion, the region surrounding the repeat was amplified. Using primerset *a-258* a PCR product of 470 bp, approximately 100 bp shorter than the expected product, was found, indicating that the deletion fell within this primerset. Likewise, amplification with primers *c* and *f* resulted in a PCR product of 160 bp in stead of the expected 260 bp. The exact breakpoint of the deletion was determined by sequence analysis. After a Centricon-100 purification step, the PCR-products obtained by amplification with primers *a-258*, were sequenced directly with primers *c* and *f*. The deletion was found to encompass part of the CGG repeat in addition to 30 bp 3′ of the CGG repeat (position 2768 to 2797 of pE5.1 [Fu et al., 1991b]). The exact sequence of the junction of the deletion is depicted in Figure 2A. Figure 2B shows a schematic representation of the deletion, in comparison with previously described deletions in the CGG repeat region.

Considering the absence of hypermethylation of the fragment with the deletion, normal mRNA expression could be expected in the cells containing the deletion as would be in line with unmethylated premutation alleles. Additionally, the finding that the deletion only included part of the untranslated region suggested that the cells containing the deletion could still produce the *FMRP*. To verify this, we performed immunocytochemistry on cultured blood lymphocytes and blood smears. Figure 3A illustrates the FMRP expression in the cytoplasm of cultured lymphocytes derived from a healthy individual. Fragile X patients with a methylated full mutation only, lack expression of *FMRP* in blood lymphocytes (Fig. 3B). In agreement, most lymphocytes (72%) derived from this patient were negative (Fig. 3C), demonstrating the silencing of the *FMR1* gene. How-

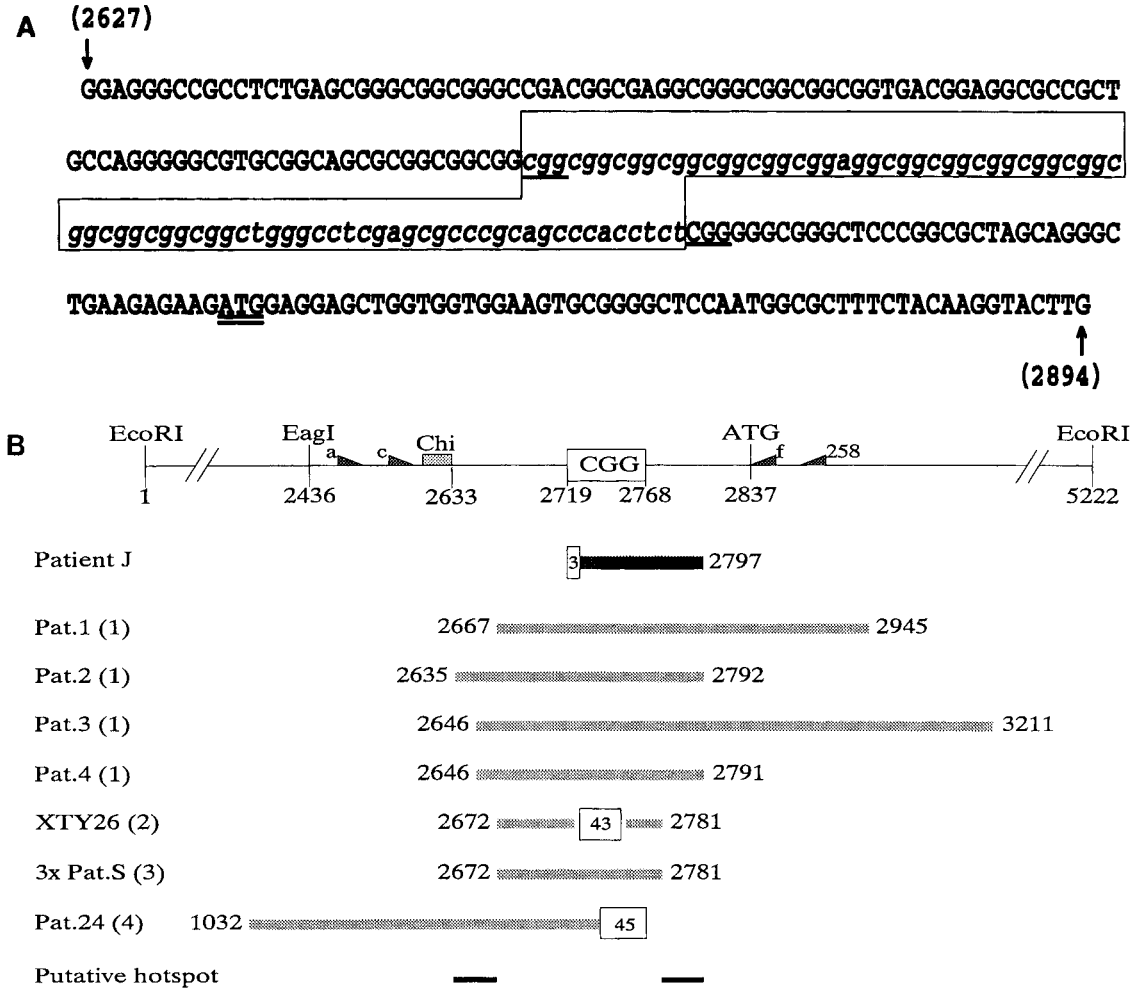

Fig. 2. **A:** Junction sequence of the deletion in patient J. The deleted sequence is given in bold lowercase. The short direct repeat flanking the deletion is underlined, the startcodon is double underlined. Depending on which short CGG repeat is deleted either three or four CGG copies remain. **B:** Schematic representation of the deletions found thus far in the CGG region (not drawn to scale). Triangles represent the primers, boxed numbers represent the number of remaining CGG copies; ATG, start codon for translation; Chi, chi-like element. Deletions described previously are as follows: 1): De Graaff et al. [1995a], 2): Kremer et al. [1991], 3): Snow et al. [1993] and 4): Meijer et al. [1994], respectively.

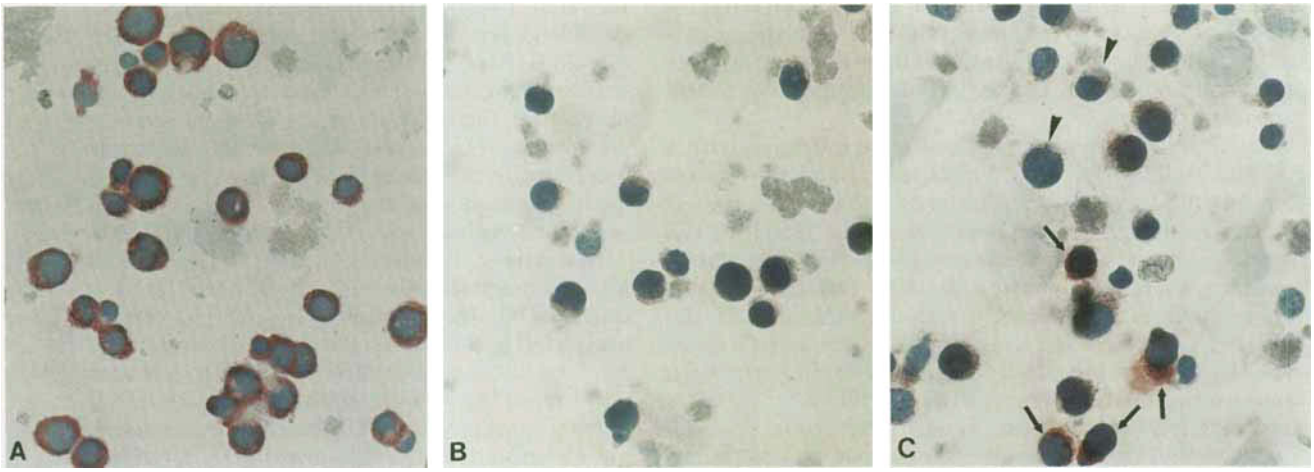

Fig. 3. FMRP expression in cultured lymphocytes in a healthy individual (**A**), a fragile X patient with a methylated full mutation (**B**), and patient J (**C**). Arrows denote FMRP positive lymphocytes, whereas the arrowheads indicate some unlabelled lymphocytes.

ever, normal cytoplasmatic expression of *FMRP* could be detected in 28% of the cultured lymphocytes. These cells are likely to contain the 100 bp deletion instead of the methylated full mutation, thus indicating that the deletion did not impair *FMRP* translation. In a blood smear of patient J, a similar percentage of the lymphocytes was found to produce the *FMRP* (data not shown), indicating that both blood smears as cultured lymphocytes can be used for the detection of *FMRP* in blood cells.

DISCUSSION

Little is known about regulatory elements in the promoter region of *FMR1*. Recently, using a *FMR1*/β-galactosidase fusion gene in transgenic mice, a 2.8 kb fragment in the 5′ region of *FMR1* was demonstrated to be sufficient for normal *FMR1* expression [Hergersberg et al., 1995]. Therefore, this 2.8 kb *Eco*RI-*Nhe*1 fragment, including the CGG repeat and a large part of the first exon, contains all regulatory elements for normal *FMR1* gene expression. In addition, a 1.6 kb deletion, coinciding with the 2.8 kb fragment, was described in a fragile X patient lacking *FMR1* mRNA expression [Meijer et al., 1994], indicating that regulatory elements reside in this fragment.

The absence of expression of *FMRP* correlated completely with methylation of the CGG repeat and the preceding CpG island of *FMR1*. In most fragile X patients with a full mutation all CpG dinucleotides in the promoter region are methylated [Hansen et al., 1992; Hornstra et al., 1993]. Recently, we described a lung tumor with a premutation allele that expressed the *FMRP* despite the methylation of the *Eag*I, *Bss*HII and *Sac*II restriction sites [De Graaff et al., 1995b]. It is very likely that not all CpG dinucleotides in this tumor are methylated. Hwu et al. [1993] performed in vitro methylation studies using different methylases. Methylation of all CpGs, using *Sss*I methylase, resulted in the total absence of the *FMR1* expression, whereas methylation with *Hpa*II methylase did not hamper the promoter activity. Thus, certain CpG dinucleotides in the promoter region appear to be essential for promoter activity of *FMR1* and these are presumably unmethylated in the tumor.

Since the deletion, observed in part of the cells in this patient, did not impair the production of *FMRP*, the 30 bp immediately 3′ of the CGG repeat do not contain any regulatory sequences. Recently we described four unrelated patients, also mosaic for a full mutation and a deletion encompassing the entire CGG repeat plus flanking sequences [De Graaff et al., 1995a]. In two patients, patient 2 and 4, the 3′ breakpoint of the deletion is located near the observed breakpoint in the current case (Fig. 2B). However, these two patients lacked, respectively, 85 bp and 74 bp immediately 5′ of the CGG repeat as well. Unfortunately the expression of *FMRP* could not be tested in these patients, for this could indicate whether regulatory sequences are present immediately 5′ of the CGG repeat.

The instability of the expanded CGG repeat is a well-described phenomenon and is present both meiotically and mitotically. Carriers of a full mutation display a large variety in repeat length, visible as a smear on Southern blot analysis [Yu et al., 1991; Oberlé et al., 1991; Wörhle et al., 1992; Devys et al., 1992]. Patients have been described to be mosaic for a full mutation in combination with either a premutation [Oberlé et al., 1991; Rousseau et al., 1994a; Nolin et al., 1994], normal allele [Van den Ouweland et al., 1994] or deletion [De Vries et al., 1993; De Graaff et al., 1995a]. The occurrence of deletions in the CGG repeat region of *FMR1* indicate that the instability is not restricted to the repeat itself, but it expands to the flanking region [Kremer et al., 1991; Meijer et al., 1994; De Graaff et al., 1995a].

Although the CGG repeat length of the mother is unknown, it is very likely that the deletion occurred within an expanded repeat. The patient has a full mutation indicating that the mother will be a carrier of either a premutation or a full mutation. In addition, the occurrence of repeat sizes below 10 CGGs is very rare,

with 6 repeats being the smallest allele found thus far [Fu et al., 1991a]. Whether this deletion occurred in a full mutation or a premutation allele depends mainly on the timing repeat expansion of premutation to full mutation.

The finding that sperm cells of male patients, with a full mutation in their blood cells, only have a premutation, resulted in two hypotheses on the possible timing of the repeat expansion [Reyniers et al., 1993]. In the first hypothesis, the repeat expands during postzygotic proliferation in the somatic cells, after the separation of the germ line. Occasionally somatic cells escape the CGG repeat expansion, thus resulting in a mosaic genotype. In this hypothesis, the deletion could occur both before amplification, thus in a premutation, or after amplification of the repeat, in a full mutation.

The second hypothesis assumes expansion of the repeat in the oocyte, during meiosis, resulting in a full mutation in all somatic cells. In some cells gametic and somatic, regression to a premutation can occur and due to a strong selection mechanism only sperm cells with a premutation can proliferate. Although *FMRP* is highly expressed during gametogenesis [Devys et al., 1993; Bächner et al., 1993], *FMRP* is not essential during gametogenesis [Bakker et al., 1994; Meijer et al., 1994]. Therefore, absence or presence of *FMRP* can not be the basis of a selection mechanism. A possible selection mechanism could be based on the finding that replication of a full mutation allele is delayed as compared to premutation alleles [Hansen et al., 1993]. Sperm cells carrying a premutation may therefore proliferate faster than sperm cells carrying a full mutation. Regression of the full mutation can not only delete CGGs but sometimes some flanking sequences as well, resulting in deletions as is found in this patient and previous reports [Kremer et al., 1991; Meijer et al., 1994; De Graaff et al., 1995a].

Regression from a full mutation to the deletion, not only involves deletion of DNA sequences but also demethylation. Methylation of the CGG repeat and CpG island may be an active process, maintained by the presence of a full mutation. Disappearance of the full mutation would result in passive demethylation. Absence of this active methylation process can thus explain the occurrence of unmethylated full mutations. On the other hand, the possibility that demethylation is an active process in itself, may not be excluded. Active removal of the methyl-groups might then result in damage to the DNA, thus causing the regression.

The fragile X syndrome is widely accepted to be caused by the absence of expression of *FMRP*. Evidence is now accumulating that a certain threshold of *FMRP* expression is required for normal cognitive functioning. Premutations are not methylated and will therefore result in a normal expression of *FMR1* [Feng et al., 1995a]. Nevertheless, fragile X patients, mosaic for a full mutation and a premutation, are not less mentally impaired as patients with a full mutation only [Rousseau, et al., 1991; De Vries, et al., 1993; Rousseau, et al., 1994a], suggesting that the percentage of cells expressing the FRMP is too low in these patients. In keeping with this, patient J, described in this paper, was found to be moderately retarded, despite 28% of the blood lymphocytes still expressing *FMRP*. Therefore 28% of the cells expressing *FMR1* appears to be insufficient for normal cognitive functioning. It should be noted that the analysis of the CGG repeat is generally performed in blood cells, which may not be a proper indication for the number of cells expressing *FMRP* in brain [Rousseau, et al., 1991; Devys, et al., 1993]. However, for obvious reasons this will be difficult to verify.

In contrast, Rousseau et al. [1994b] described a cognitively normal male with an unmethylated full mutation in 60% of the lymphocytes. Although no data were presented on *FMR1* expression, it is likely that the 60% cells containing an unmethylated full mutation, *FMRP* is expressed. Apparently this amount is sufficient for a normal cognitive function. Normal males were found to have a unmethylated full mutation of up to 1,500 CGGs in all cells [Smeets et al., 1995]. In contrast to the finding by Feng et al. [1995b], who described the absence of FMRP translation of alleles with CGG repeats above 200, Smeets et al. [1995] found protein expression in all cells, although at reduced levels. This may suggest that both a reduced expression of *FMRP* in all cells and normal expression of *FMRP* in a high percentage of cells do not impair cognitive functioning. Noteworthy in this respect is the finding that approximately 60% of the females carrying a full mutation have some degree of mental impairment. This is in striking contrast to other X-linked disorders. A possible explanation for this phenomenon is, at least 50% of the cells need to express the *FMRP* for a normal cognitive function. Hence, in females, skewed X inactivation, with over 50% of the active X chromosomes containing the methylated full mutation, may lead to less than 50% of the cells expressing FMRP, thus leading to mental impairment. This would also imply that, if in future gene therapy is considered, one should keep in mind that a very high percentage of cells needs to be targeted.

We recently described a putative hotspot region for deletions 5′ of the CGG repeat, suggesting that at the 5′ end of the CGG repeat some elements are present that might lead to the instability [De Graaff et al., 1995a]. In addition Snow et al. [1994] mentioned to have detected a deletion in three unrelated patients coinciding with the deletion of Kremer et al. [1991], thus supporting the possible presence of such a hotspot. Indeed immediately 5′ of the deletions a chi-like element was found (Fig. 2B). At the 3′ site of the CGG repeat the breakpoints appeared to be more scattered; only 3 out of 5 deletion described were located within 10 bp. However, the 3′ breakpoint of the currently described patient is also in the vicinity of these 3 breakpoints, suggesting that there might be an additional hotspot for deletions 3′ of the CGG repeat.

In line with the deletion reported previously, the deletion in patient J is flanked by a short direct repeat, "CGG" (Fig. 2A). Short direct repeats of 2–6 bp often flank deletions and it has been suggested that these short repeats slip and mispair during replication, resulting in the formation of a loop. Subsequent excision of this loop removes both the sequences between the repeats as well as one of the repeats [Canning et al., 1989;

Thacker et al., 1992]. Depending on which "CGG" repeat was deleted, either three or four CGG copies of the repeat remained.

Initially, blood smears could not be made within 6 hr after bleeding, making it impossible to perform the immunolabeling directly on the blood smears [Willemsen et al., 1995]. To overcome this problem, lymphocytes were first cultured according to standard conditions for cytogenetic expression [Verkerk et al., 1992]. Later, a second blood sample was taken from the patient, which was immediately used for making a blood smear. An equal percentage of FMRP expressing lymphocytes was found in both cultured lymphocytes and the blood smear. This implies that if blood smears can not be made within 6 hr after bleeding, the antibody test can be performed on cultured lymphocytes.

In conclusion, we have described a male fragile X patient, mosaic for a full mutation in combination with a deletion of the CGG repeat and 30 bp immediately flanking the 3′ site of the CGG repeat. This deletion did not impair the production of *FMRP*, indicating that the deleted region did not contain regulatory elements required for expression of *FMR1*. Assuming that the pattern of mosaicism in brain is comparable to the pattern in lymphocytes, it can be concluded that expression of FMRP in 28% of the cells is insufficient for normal cognitive functioning.

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